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A novel L-ascorbic acid and peptide conjugate with increased stability and collagen biosynthesis

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L-ascorbic acid (Vitamin C) and peptide are both useful compounds for collagen biosynthesis in cosmeceuticals (cosmetic and pharmaceutical fields). The instability of these compounds, however, limit their application in these industries. In this report, we describe the development of a novel compound, Stabilized Ascorbyl Pentapeptide (SAP), which physically is much more stable than L-ascorbic acid in water. The inhibitory effects of this SAP compound on tyrosinase and melanin synthesis is comparable to that of L-ascorbic acid. Importantly, the SAP compound displays no cytotoxicity at a high concentration (5 mM). The ability of SAP to promote collagen biosynthesis is greater than that of L-ascorbic acid or the KTTKS peptide alone. Considering the *in vitro* stability and functional effects, our data strongly suggest that the SAP compound is a good candidate not only as a cosmetic ingredient, but also as a wound healing agent. [BMB reports 2009; 42(11): 743-746]

INTRODUCTION

The concerns of skin aging are increasing important to the fields of dermatology and cosmetic medicine. Skin aging is a complicated progress caused in part by the reduction of ECM components including laminin, fibronectin, collagen, etc (1). Twenty types of collagen are known to exist and are the major component of the ECM in humans. Type I collagen is the most abundant type of collagen and functions in structural integrity, cell adhesion and migration, tissue remodeling, and wound healing (2, 3). These characteristics have led researchers to focus on the importance of collagen in the aging process. In 1993, Kou Katayama et al. identified that a pentapeptide (Lys-Thr-Thr-Lys-Ser) from C-peptides of type I procollagen has the ability to promote collagen biosynthesis (4). This group suggested that precursor molecules containing NH_2 and COOH propeptides that were cleaved off extracellularly by specific peptidases may be involved in the regulation of collagen biosynthesis.

It is well known that L-ascorbic acid also functions in promoting the biosynthesis of collagen, a key structural protein of the skin. The addition of ascorbic acid to cultured human dermal fibroblasts not only remarkably increased the effects of the collagen biosynthesis but also reduced skin damage from free radicals (5, 6). When ascorbic acid is properly delivered to skin cells, damaged skin may be improved and strengthened during skin regeneration (7). The stability of ascorbic acid is so low, however, that it is difficult to deliver and does not remain in skin tissue for extended periods of time. According to previous reports, ascorbic acid is rapidly oxidized by air in aqueous solutions (8). Historically, retinoic acid, also known as a potent inducer of the collagen biosynthesis, has been used as a key cosmetic material commercially (9). Unfortunately, overdoses of retinoic acids, a family of lipid-soluble vitamins, are known to have negative side effects in pregnant women and children (10).

Here, we synthesized various L-ascorbic acid and peptide conjugates and identified the conjugated form of pentapeptide and ascorbic acid that most effectively increases collagen synthesis and ascorbic acid stability *in vitro*. Our novel peptide conjugate is a potent anti-wrinkle agent and will expand the use of peptides in the cosmeceutical field (11).

RESULTS

Increased *in vitro* stability of SAP

It is well known that L-ascorbic acid is unstable and readily oxidized to dehydro-L-ascorbic acid. To improve the *in vitro* stability of L-ascorbic acid, it is modified by attaching a phosphate group to the ribose ring. To activate collagen synthesis *in vivo*, L-ascorbic acid is coupled with a peptide (Supplement Fig. 1). While L-ascorbic acid is nearly completely decomposed within 3 days, SAP remains stable for more than 28 days in distilled water at room temperature (Fig. 1).

Inhibition of tyrosinase activity

The ability of SAP to inhibit tyrosinase activity was measured *in vitro*. SAP has one quarter to one half the tyrosinase inhibiting activity of Kojic acid, one of the most potent tyrosinase inhibitors in enzymatic assays (Fig. 2). As shown in Fig. 2, the ability of SAP to inhibit tyrosinase activity is comparable to

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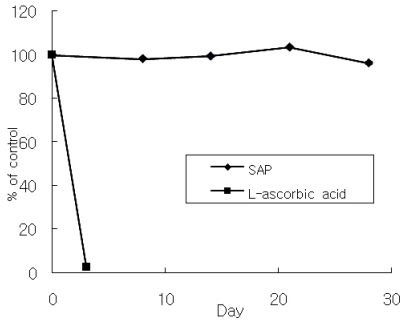

Fig. 1. *In vitro* stability of SAP and L-ascorbic acid. The *in vitro* stability of SAP is dramatically increased in distilled water at room temperature compared to L-ascorbic acid.

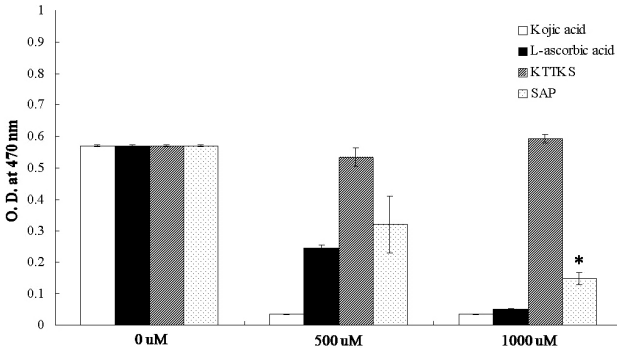

Fig. 2. Inhibition of tyrosinase activity by SAP. The inhibitory effect of SAP on tyrosinase activity as measured with the *in vitro* assay is not significantly different than that of L-ascorbic acid at the tested concentrations. The values are expressed as mean ± S.E. from three independent experiments. Kojic acid, positive control. *P < 0.5 compared with the L-ascorbic acid only treatment.

that of ascorbic acid at 500 uM and 1,000 uM. In addition, the tyrosinase inhibition activity of SAP is concentration-dependent, a characteristic which suggests that a tyrosinase-inhibitory effect comparable to that of kojic acid can be obtained at higher concentrations.

Inhibition of melanogenesis

To determine whether SAP also functions as an inhibitor of melanin synthesis, B16 murine melanoma cells, cells which actively produce melanins were used. These cells were treated with various compounds at a range of concentrations in order to evaluate their impact on melanin synthesis. As shown in Fig. 3a, SAP has a melanogenesis-inhibitory activity more than 10% higher than arbutin, a compound known to inhibit melanogenesis at a concentration of 1 mM. In addition, at 1 mM, SAP is a more potent inhibitor of tyrosinase activity than L-ascorbic acid at the same concentration. A naked-eye view of cell pellets following treatment indicates that SAP-treated cells have decreased amounts of melanin compared to control (no

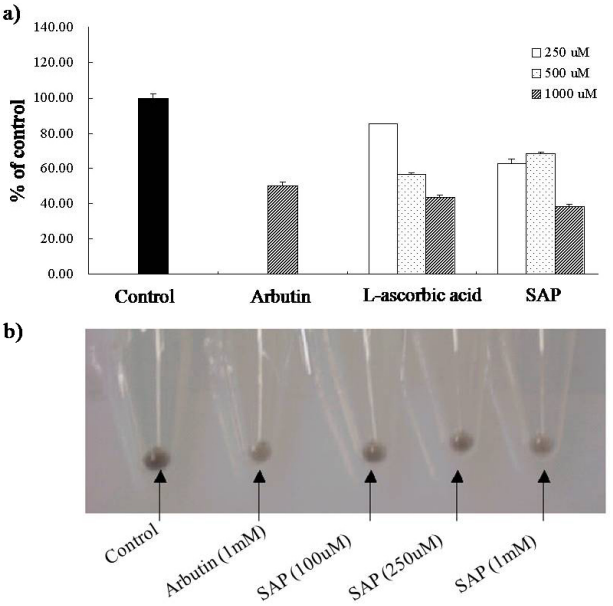

Fig. 3. Inhibition of melanogenesis by SAP. (a) The melanin contents of B16 cells were significantly reduced following treatment with SAP compared to control cells (no treatment) at the tested concentrations. The degree of melanin synthesis-inhibition following SAP treatment is comparable to that of L-ascorbic acid and arbutin (positive control) at 1 mM. The values are expressed as mean ± S.E. from three independent experiments performed in duplicate. (b) Representative photograph showing melanin contents after inhibition with SAP at the tested concentrations and cell pelleting.

treatment) or arbutin-treated cells (Fig. 3b).

Collagen biosynthesis assay

To evaluate the ability of various compounds to stimulate collagen biosynthesis, human dermal fibroblasts (HDF) were treated with L-ascorbic acid, SAP, or pentapeptide (KTTKS) at various concentrations for 72 hours (Fig. 4). At lower concentrations (1-10 uM), the ability of SAP to stimulate collagen biosynthesis is comparable to that of the L-ascorbic acid treatment. At a higher concentration (100 uM), however, SAP-treated cells displayed a more than 100% increase in collagen biosynthesis compared to L-ascorbic acid-treated cells. High concentrations of L-ascorbic acid may not be as effective as SAP at stimulating collagen biosynthesis because it is degraded more quickly in the media. KTTKS treatment alone has no significant effect on collagen biosynthesis. The reduced ability of the peptide alone to impact collagen biosynthesis may be because it inefficiently penetrates the cell and/or is degraded by peptidases within the cell and culture media.

SAP is not cytotoxic to HDF cells

Results of the MTT assay indicate that the activity of mitochondrial dehydrogenases do not decrease following incubation of human dermal fibroblasts (HDF) with various concentrations (1

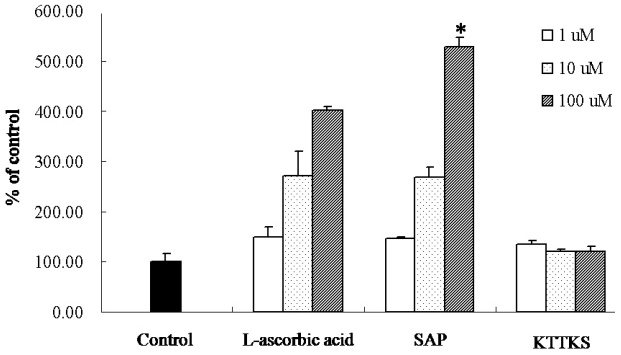

Fig. 4. Collagen biosynthesis induced by SAP. The amount of collagen synthesis following SAP treatment is more than 4 times than that of the control (no treatment) at 100 uM. The collagen synthesis activity of SAP is higher than those of individual treatments with L-ascorbic acid and KTTKS at 100 uM. The values are expressed as mean ± S.E. from three independent experiments performed in duplicate. *P < 0.05 compared with the L-ascorbic acid only.

uM-5 mM) of L-Ascorbic acid or SAP for 72 hours (Supplemental Fig. 2). It is well known that L-ascorbic acid protects cells from cytotoxic damage and thus it is not expected to be cytotoxic. At concentrations up to 5 mM, SAP-treatment results in only a mild increase of the mitochondrial dehydrogenase activity, not cytotoxicity; these results confirm the safety of this compound.

DISCUSSION

L-ascorbic acid (Vitamine C) can not penetrate the outer layer of skin because of its hydrophilicity. In addition, because of its low stability against oxygen, light and heat, it has been difficult to develop and maintain the effective activity of L-ascorbic acid for commercial use in the cosmetics field (8). As ascorbic acid is highly labile to environmental conditions, it requires modification to reduce rapid degradation. To overcome these problems, there are some alternatives, including modification with several fatty acid-bound complexes or treatment with a mixture of zinc sulfate and L-tyrosine with L-ascorbic acid, and L-ascorbate-2-phosphate. It remains necessary to explain the mode of action of these treatments (12, 13). For these reasons, the development of stabilized ascorbic acid is important. We have developed a novel ascorbic acid-peptide conjugate in which a peptide is linked with L-ascorbic acid via a succinoyl linker. The increased stability of our conjugate may be attributed to not only the phosphate in the L-ascorbic acid ring (leading to the stabilization of the double bond in the ring), but also the peptide-specific hydrogen bonding of the conjugate (12). Given that the compound described is non-cytotoxic and stable, as shown in Fig. 1 and Supplement Fig. 2, it may be used as a tyrosinase inhibitor instead of kojic acid, a compound known to be carcinogenic (14).

As shown in our Fig. 4, neither ascorbic acid or pentapeptide are as active as SAP in promoting collagen biosynthesis. These results may indicate that the linker is indispensible for causing strong collagen expression in human fibroblast cells. One possible explanation for why SAP had the biggest effect on collagen synthesis at high concentration is the unique impact of the ester bond on the succinoyl linker of SAP. This linker is highly labile to esterase, and the activity of this enzyme results in the release of both L-ascorbic acid and the pentapeptide into the cell. These products likely function synergistically to effect collagen biosynthesis, anti-oxidation, and the inhibition of melanin synthesis.

Recently, we have shown that the prototype of our compound can increase collagen production in cells and is more stable than L-ascorbic acid in rat skin extracts. These characteristics suggest that the SAP may be a useful cosmeceutical material (15). Here, we describe the activity of a novel compound, which is not only physically stable, but which also displays anti-aging, wound-healing, and skin-whitening activities.

In conclusion, we have presented the first promising experimental evidence that ascorbic acid-conjugated peptide is a more stable useful cosmetic and pharmaceutical material *in vitro* and *in vivo*. Further studies will be focused on characterizing various formulations of our conjugate for their anti-aging and anti-wrinkle acivities through clinical applications.

MATERIALS AND METHODS

Synthesis of the SAP compound and the *in vitro* stability test

SAP was synthesized by modifying the strategy (16) described in our previous report (15). The linear peptide KTTKS was synthesized on a 2-chlorotrityl resin preloaded with Fmoc-serine using standard Fmoc chemistry employing HBTU/HOBt as a coupling reagent. Next, the peptide was coupled with a succinoyl phosphoryl ascorbate derivative to generate SAP. The *in vitro* stability test was performed by dissolving 0.1 mg Vitamin C and SAP in distilled water at room temperature and analyzed by HPLC for 28 days.

Tyrosinase inhibition assay

Each sample (kojic acid, L-ascorbic acid, KTTKS, SAP) was individually mixed with Mushroom tyrosinase (1,500-2,000 u/ml, Sigma T3824) in 0.1 M phosphate buffered saline (pH 6.5). 40 ul of 1.5 mM tyrosine (Sigma, T8566), used as an enzyme substrate, was added to each mixture and incubated for 10-15 minutes at 37°C in a 5% CO_2 incubator. The optical density (O.D.) of the enzymatic product, Dopachrome, was measured at 474 nm using an ELISA reader.

Measurement of melanin contents

B16 murine melanoma cells (50,000-100,000) were seeded to 24 well plates in DMEM (Gibco, USA) containing 1% FBS (Invitrogen, Maryland, USA) and were incubated at 37°C in a 5% CO_2 incubator for 24 hrs. Test samples were treated at various concentrations and incubated at 37°C in a 5% CO_2 incubator for 72 hrs. After incubation, cells were lysed with 1 ml

of 1 N NaOH, and 200 ul of each aliquot was transferred to a 96 well plate. O.D. values were measured at 405 nm using an ELISA reader. Melanin contents were expressed as a percentage of untreated control.

In vitro cytotoxicity assay

Human dermal fibroblasts were incubated at 37°C in a 5% CO_2 incubator for 72 hrs with various concentrations of test samples ranging from 1 uM to 5,000 uM to identify the cytotoxicity of test samples. Mitochondrial dehydrogenase activity was used as an index of cell viability and was evaluated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma), which is reduced to an insoluble formazan dye. Culture media was replaced with fresh 0.1% FBS media containing 1 mg/ml of MTT and incubated for 3-4 hrs. Next, DMSO (200 ul) was added to each well. After incubating the microtiter plates at room temperature for an additional 30 minutes, O.D. values were measured at 490 nm using an ELISA reader.

Measurement of collagen biosynthesis

Human dermal fibroblasts (HDF) were seeded to 96-well plates (3,000 cells per well) and grown in DMEM containing 5% fetal bovine serum (Invitrogen, Maryland, USA) for 24 hours. Next, the media was replaced with fresh assay media containing 0.1% FBS and the corresponding treatment to test for collagen production. After incubation for 72 hours, equal amounts of supernatant were added to a plate coated with human collagen type I antibody. The plate was incubated at room temperature for 2 hours to allow for reaction of antibody and antigen. Next, the plate was washed three times with phosphate buffered saline containing 0.5% Tween 20 to remove unbound collagen, followed by the addition of biotin-labeled human collagen type I antibody to each well. Roughly an hour later, streptavidin-horseradish peroxidase (St Louis, Sigma) was added to each well to detect biotin-labeled antibodies bound to type I collagen. The quantity of collagen in each well was converted into O.D. values by using tetramethylbenzidine (St Louis, Sigma) as a substrate of HRP. The reaction between HRP and TMB was stopped by the addition of 1N HCl, and the O.D. value was measured at 450 nm.

Statistics

Statistical significance was evaluated by using student's t-test.

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